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(71) Applicant: SHIONOGI & CO., LTD.
12, Dosho-machi 3-chome Higashi-ku
Osaka 541(JP)

(72) Inventor: Teraoka, Hiroshi
2-47-10, Takakuradai
Sakai-shi Osaka(JP)

(72) Inventor: Sato, Kosaburo
5-15, Koshienabiki-cho
Nishinomiya-shi Hyogo(JP)

(72) Inventor: Tanaka, Kentaro
3-19-2, Furuedai
Suita-shi Osaka(JP)

(74) Representative: Hranitzky, Wilhelm Max et al,
NOVAPAT - CABINET CHEREAU 5, Place du Molard
CH-1204 Genève(CH)

(54) **Novel interferon alphas.**

(57) Novel IFNαS51B10 and IFNαS17H9 of this invention are prepared from BALL–1 cell induced with Sendai virus according to the well known recombinant DNA technique. Further, this invention relates to a DNA encoding interferon αS51B10 or αS17H9, a recombinant plasmid enabling an expression of interferon αS51B10 or αS17H9 in a host microorganism and a microoraganism transformed by the recombinant plasmid. These two IFNαs have antiviral and anti-tumor activity as other subtypes of IFNα and are useful as medicines for human and animal.

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NOVEL INTERFERON ALPHAS

(1) Field of the Invention

This invention relates to novel interferon alphas, namely interferon αS51B10 and interferon αS17H9. Further, it relates to DNA sequence and recombinant plasmid enabling an expression of these interferons and a microorganism transformed by the plasmid. The above interferon alphas each has antiviral and antitumor activity and is therefore utilized as a medicine for human and animal.

(2) Description of the Prior Art

Human interferon (hereinafter referred to as IFN) has α, β, and γ type, all of which are (glyco)proteins having antiviral activity and other broad physiological activities (W. E. Stewart II: The IFN System, Springer-Verlag, New York-Wien 1979).

It is well known that especially IFNα has many subtypes (S. Pestka: Arch. Biochem. Biophys 221, 1-37 (1983); C. Weissmann et al: Interferon, UCLA Symposia on Molecular and Cellular Biology 25, 295-326 (1982), Academic Press), and their antiviral, anti-cell proliferation and NK-activating activities are fairly different from each other's (E. Rehberg et al, J. Biol. Chem. 257, 11497 (1982)).

Leukocyte, Namalva cell, KG-1 cell and the like are recognized as producing a large amount of IFNα. From these cells mRNAs are extracted and the genes encoding subtypes of IFNα are isolated through cDNA cloning. However, the proportion of the amount of the subtypes containing is different in the each cell (I. Hiscott et al, Nucl. Acids. Res. 12, 3727-3746 (1984)).

Miyoshi et al found that BALL-1 cell isolated from leukemia leukocyte (I. Miyoshi et al, Nature 267, 843-844 (1977)) produces a lot of IFNα (Miyoshi et al, Progress in medicine (Igaku no

ayumi) 113, 15-16 (1980)).

Novel IFNα S51B10 and IFNα S17H9 of this invention are prepared from BALL-1 cell induced with Sendai virus according to the well known recombinant DNA technique. Further, this invention relates to a DNA encoding interferon α S51B10 or α S17H9, a recombinant plasmid enabling an expression of interferon α S51B10 or α S17H9 in a host microorganism and a microorganism transformed by the recombinant plasmid. The IFNα S51B10 and IFNα S17H9 have DNA sequences, as shown in Fig.2 and 3, respectively, different from those of all the already known subtypes of IFNα and so they are recognized as new. These two IFNαs have antiviral and anti-tumor activity as other subtypes of IFNα and are useful as medicines for human and animal.

Fig. 1-3 show the DNA sequence and the corresponding amino acid sequence of IFNα S80A2, IFNα S51B10 and IFNα S17H9, respectively.

Fig. 4-6 show the restriction map of cDNA of IFNα S80A2, IFNα S51B10 and IFNα S17H9, respectively, prepared from mRNA derived of BALL-1 cell.

Fig. 7 shows the recombinant plasmid and the plasmid and vector used for the preparation thereof.

Fig. 8 shows the change of the production of IFN by BALL-1 cell with the passage of time.

Fig. 9 shows the recombinant plasmid for the expression of IFNα S17H9 or IFNα S51B10 and the plasmid and vector used for the preparation thereof.

In order to discover novel IFN having potent IFN activity the

inventors cultured BALL-1 cell and provided cDNA bank by well-known recombinant DNA technique, from which are isolated cDNAs of 2 types of IFNα different from every known type of IFNα. And the recombinant plasmids enabling the expression of the corresponding IFNαs were made by using these cDNAs. Then we transformed a microorganism with these plasmids and succeeded to produce the desired novel 2 types of IFNα.

A lot of complementary DNA (cDNA) clones were prepared by using as template IFNα mRNA separated from BALL-1 cell. Three clones were isolated from these clones, and one of them was already known one and other two clones were recognized as new from their base sequence and the amino acid sequence encoded thereby. The IFNs expressed by 3 clones were named IFNαS80A2, IFNαS51B10 and IFNαS17H9, respectively. The DNA sequences encoding these IFNs are shown in Fig.1, Fig.2 and Fig.3 and the amino acid sequence of each IFNα deduced from the DNA sequence is shown under the DNA sequence in each Fig. Of course, every amino acid is of L type and represented in Fig.1-3 by one letter defined by International Union of Biochemistry. What the letter means is as follows.

A: Alanine,	C: Cysteine,	D: Asparatic acid,
E: Glutamic acid,	F: Phenylalanine,	G: Glycine,
H: Histidine,	I: Isoleucine,	K: Lysine
L: Leucine,	M: Methionine,	N: Asparagine,
P: Proline,	Q: Glutamine,	R: Arginine,
T: Threonine,	V: Valine,	W: Tryptophan,
Y: Tyrosine.		

The restriction map by main restriction enzymes of each cDNA of IFNαS80A2, IFNαS51B10 and IFNαS17H9 derived from mRNA of BALL-1 cell is shown in Fig.4, Fig.5 and Fig.6.

As shown in Fig.1, the base sequence of IFNαS80A2 is the same as IFN-α-N reported by E. Gren et al (J. IFN Research 4, 609-617 (1984)).

IFNαS51B10 (Fig.2) is very similar to IFNαG (Goeddel et al Nature 290, 20-26 (1981)) and IFNα5 (Weissman, the same as noted above). A part of the base sequence of αG has not yet been elucidated and the 33 amino acid residues from N terminal cannot be presumed. Therefore, it is impossible to determine if IFNαS51B10 is the same as αG. Since as to α5 the amino acid sequence only has been reported, compared with the amino acid sequence of αS51B10, the sole difference is recognized at 51th amino acid which is Lysine in α5 but Alanine in αS51B10. However, αS51B10 produced by E.coli shows antiviral activity in mouse cells but α5 produced by E.coli in the similar manner does not show antiviral activity in mouse cells (Nagata et al. Abstract of Japanese Virus Congress, 130, 1984; Proc. Natl. Acad. Sci. USA, 81, 5056-5090 (1984)). Thus, the chemical structure of subtype S51B10 of IFNα is similar to that of known α5, but a remarkable difference is recognized in physiological activity, and so IFNαS51B10 is determined to be novel IFNα.

IFNαS17H9 (Fig.3) resembles known α8. Though all subtypes of IFNα consist of 166 amino acid residues except α2 (or αA) consisting of 165 amino acid residues (S. Pestka, the same as noted above, Weissmann, the same as noted above), this subtype consists of 161 amino acid residues. Therefore, it is recognized as novel IFNα.

By using cDNAs of IFNαs of this invention the recombinant plasmid expressing each IFNα is prepared according to well known recombinant DNA technique. The plasmid provided is introduced into an appropriate microorganism to give a transformed microorganism.

Desired IFNα is produced by this microorganism. This invention comprehends IFNα S51B10 and IFNα S17H9 produced by this serial method, recombinant plasmids expressing them and microorganisms transformed with these plasmids.

Reagents, methods and operations used in the production of the desired materials as mentioned above are shown below. However, the present invention is not limited by these disclosures. In the following disclosure "IFNαs" is used as a general term of IFNα S80A2, IFNα S51B10 and IFNα S17H9.

I Preparation of cDNA

i Reagent and Method

A. Used Microorganism

A used microorganism is already known Escherichia coli K-12 such as HB101, χ1776, JM103, C600 and so on, Bacillus subtilis such as Maburg 168, Saccharomyces cerevisiae and the like. These microorganisms are available from authorized depositories such as American Type Culture Collection.

These microorganisms conform to Japanese guide line for recombinant DNA experimentation and this experiment was carried out according to the experimental guide line.

B. Used Enzymes, Reagents and Methods

Several kinds of restriction enzymes, DNA polymerase, T4 kinase, S1 nuclease, terminal deoxynucleotidyl transferase, reverse transcriptase, RNase H, DNA ligase and so on are all on the market. Human placenta RNase inhibitor is prepared in accordance with Blackburn's method (P.Blackburn, J. Biol. Chem. 254, 12484-12487(1979)). Plasmid DNA and vector DNA on the market can be used. Recombinant plasmid of this invention is prepared by the standard alkali-SDS method (Birnboin et al, Nucl. Acids, Res. 7, 1513-1523 (1979)) and purified with CsCl. Sequencing of DNA is

achieved by chain termination method of Sanger et al using M13 phage (F. Sanger et al, Proc. Natl. Acad. Sci, USA 74, 5463-5467 (1977)). Other general recombinant DNA techniques are detailed in and conform to Methods in Enzymology (Recombinant DNA), Vol.68 (part A), Vol.100 (part B) and Vol.101 (Part C).

C. Chemical Synthesis of Oligodeoxynucleotide

Oligodeoxynucleotide can be synthesized by using dideoxynucleotide as constitution block (Broka et al, Nucleic Acids Res. 8, 5461-5471 (1980)) according to improved phosphotriester solid phase synthesis (Miyoshi et al, Nucleic Acids Res. 8, 5491-5505 (1980)). Material for the synthesis and the general method noted in Miyoshi et al, Nucleic Acids Res. 8, 5507-5517(1980) are preferably used.

Oligonucleotide used as adaptor noted later is provided by linking specified dinucleotide or mononucleotide to 5' terminal. Mixed probe disclosed later is synthesized according to the method of Ike et al. (Nucleic Acids Res. 11, 477-488 (1983)).

i Operation

Usual genetic operation can be applied to the preparation of cDNA encoding IFNαs of this invention and the operation is shown below.

(a) Induction of IFNα in BALL-1 Cell

BALL-1 cell (human lymphoblastoid cell) is cultured in growth medium, primed as occasion demands and then induced with IFN-production inducer (for example, Sendai virus) so as to produce IFN.

(b) Preparation and Measurement of IFNmRNA

A change of the concentration of IFNmRNA produced in cultured cells of (a) with the passage of time is measured and when the concentration reaches to maximum the mRNA containing poly (A) is

collected from the cultured cells by phenol extract and oligo (dT) cellulose chlomatography reported by Green et al. (Arch. Biochem. Biophys. 172, 74-89 (1975)).

(c) Synthesis and Cloning of cDNA

The cDNA can be prepared from mRNA according to the usual method and preferably be prepared by cloning according to Okayama-Berg's method (Med. Cell. Biol. 2, 161-170, (1982)) developed by Okayama and Berg.

(d) Preparation of Oligodeoxynucleotide probe

The ^{32}P-oligodeoxynucleotide probe is prepared in order to search cDNA of IFNα from cDNA prepared in the above step. For example, the sequence complementary to the sequence of 62th to 77th from ATG of the DNA of each IFNα subtype is used as probe in accordance with the report of Goeddel et al. (Nature 290, 20-26 (1981)).

(e) Screening of cDNA by the Above Probe

The cDNA of IFNα is isolated by using the above probe. The isolation is preferably performed by colony hybridization (M. Grunsteins et al, Proc. Natl. Acad. Sci. USA 72, 3961-3965 (1975)).

(f) Analysis of IFNα cDNA

The clones having almost full-length IFNα cDNA are selected from the clones isolated in (e) and their restriction maps are made. The clones having restriction map different from that of already known IFNα are separated and their DNA sequences are determined to provide cDNAs of IFNα S51B10 and α S17H9. At the same time, the already known clone of IFNα S80A2 is separated and its DNA sequence is determined to prepare cDNA of IFNα S80A2.

The amino acid sequences of IFNαs of this invention are determined from cDNA sequences provided by the above procedure to

give the results shown in Fig 1-3.

This invention comprehends every DNA encoding the amino acid sequence of IFNα S51B10 or α S17H9 and is not limited to the DNA shown in Fig.2-3.

Ⅱ. Preparation of Expression Plasmid

ⅰ. Reagents and Methods

Some conditions in this step is the same as exemplified in A, B, and C of the above I and other conditions are as follow.

D. Expression Vector

Several kinds of vectors of E. coli such as, for example, lac system, Trp system, Trp-lac fusion system, main operator and promoter system of λ-phage (P_L etc.) and λ-phage reconstruction promoter (CIP_RP_L) (Tsurimoto et al, Mol. Gen. Genet. 187, 79-86 (1982)) are mainly employed. Yeast vector such as pFRPn(Hitzeman et al, Nature 293, 717-722 (1981)), Bacillus vector such as pKTH53 (Palva et al, Proc. of the Ⅳ International Symposium on Genetics of Industrial Microorganisms, (1982) 287-291) and so on can be employed, too.

E. Synthesized Oligodeoxynucleotide Adaptor

In order to express mature IFN in microorganisms it is necessary that the DNA sequence encoding signal peptide and upstream therefrom is removed from cDNA, initiation codon ATG is linked thereto and the resulting sequence is linked to promoter and introduced into microorganisms. Moreover, such the method is often used as oligodeoxynucleotide is inserted between Shine-Dalgarno (SD) sequence and ATG so that the expression amount of foreign protein is increased.

For example, in order to insert oligodeoxynucleotide causing the increase of the expression of IFNαs, in this invention the cDNA is cut by Sau3AI at between the codons encoding the first and

the second amino acid of N-terminal of mature IFNαs. Therefore, such a synthesized oligomer is prepared as having a codon TGT encoding cysteine the first amino acid which is lost by the Sau3AI digestion and an initiation codon ATG and Cla I cutting site able to link to Trp-promoter (Fig. 7(b)). The examples are shown below.

(5') CGATACATGTGT
TATGTACACACTAG(5')

(5') CGATACTATATGTGT
TATGATATACACACTAG(5')

(5') CGATATATGTGT
TATATACACACTAG(5')

(5') CGATACTATGTGT
TATGATACACACTAG(5')

(5') CGATATTATGTGT
TATAATACACACTAG(5')

(5') CGATAGCTTTATGTGT
TATCGAAATACACACTAG(5')

F. Synthesized Deoxynucleotide Oligomer for ATG Vector

When an expression plasmid is prepared by using ATG vector, synthesized deoxynucleotide oligomer prescribing SD-ATG of appropriate kinds of bases and appropriate length is inserted into an expression vector. In this invention, since the synthesized deoxynucleotide oligomer is altered according to the properties of IFNαs, it is named Sn (n = integer of 7 to 20). The expression plasmid prepared by using ATG vector into which this oligomer pair is inserted is named pSn-IFNα. Therefore, ATG vector into which is inserted the following oligomer at one end of which has Cla I cutting site in order to employ Trp-promoter (Fig.7 (b)) and at another end of which has ATG and EcoRI cohesive end thereafter is

provided to give the preferable result.

(5')	CGATACTATATG TATGATATACTTAA (5')	(n=11)
(5')	CGATACATG TATGTACTTAA (5')	(n=8)
(5')	CGATATATG TATATACTTAA (5')	(n=8)
(5')	CGATACTATG TATGATACTTAA (5')	(n=9)
(5')	CGATATTATG TATAATACTTAA (5')	(n=9)

G. Method for the Preparation of Expression Plasmid

It is preferable to be referred to the method using a synthesized oligodeoxynucleotide adaptor (Goeddel et al, Nature 287, 411-416(1980)) and the method by ATG vector (Nishi et al, DNA 2, 265-273 (1983)).

H. Insertion of Terminator

For the purpose of the increase of the production of IFN in E. coli, an already known terminator such as ribosomal RNA gene's terminator reported by Brosius et al (J. Mol. Biol. 148, 107-127 (1981), Gene 27, 161-172 (1984)) or phage gene's terminator reported by Krisch et al (Proc. Natl. Acad. Sci. USA 29, 4937-4941(1984)) is preferably inserted into the downstream of IFNα cDNA in the expression plasmid.

i Operation

(g) Preparation of Expression Plasmid

The DNA sequence (Fig. 1-3) encoding the amino acid sequence of mature IFNα in plasmid pIFNαs (Fig.4-6) provided by cloning in I is inserted into an expression vector having an appropriate promoter. The synthesized deoxynucleotide oligomer noted in E and

F is used in this operation. This operation can be carried out according to already known method as noted in G. Moreover, as occasion demands a terminator is inserted.

The preparation of the expression plasmid for IFNα S80A2 using Trp-promoter is exemplified below.

(i) Example of Using Synthesized Oligonucleotide Adapter

① The expression vector, pTrp-promoter vector (Fig. 7(b)) that promoter operator of Trp-operon and SD sequence of E.coli are inserted into plasmid pBR322 at Cla I cutting site is digested by Cla I and Acc I to provide a fragment containing promoter. Plasmid pIFNα S80A2 (Fig. 7(a)) is-digested by Acc I and Sau96 I to give a fragment having IFN structural gene, which is linked to the above fragment at Acc I .

② Plasmid pIFNα S80A2 is digested with Sau3AI to provide 176 bp fragment containing Sau96I site. This fragment is digested by Sau96I to give a mixture of 34 bp and 142 bp fragments having Sau3AI end and Sau96I end.

③ A synthetic oligodeoxynucleotide adaptor mentioned in E having initiation codon ATG and codon TGT encoding cysteine of N-terminal is synthesized.

④ The fragments and oligomer provided in above ①, ② and ③ are linked to give plasmid pTrp-IFNα S80A2.

(i) Example of Using ATG Vector

① Trp-promoter provided by digesting pTrp-promoter vector (Fig. 7(b)) with Cla I and Pst I , pBR322 digested by Pst I and EcoRI and a synthesized oligomer mentioned in above F are linked to provide ATG vector (Fig. 7(d)).

② ATG vector is digested with EcoRI and S1 nuclease and then with Pst I to give Trp-promoter fragment having Pst I cohesive end, SD-ATG prescribed by Sn and ATG flush end.

③ Next, the plasmid from which the gene encoding signal peptide and the nucleotide sequence upstream therefrom in terms of a transcripting direction are removed and which carries IFNα cDNA is provided by employing synthesized deoxynucleotide oligomer ((5') CGAAGCTTGT and (5')GATCACAAGCTT). This oligomer complements the codon TGT which encodes cysteine of N-terminal amino acid of mature IFNα and which is lost by Sau3AI digestion, and introduces HindⅢ cutting site just before the codon (Fig. 7 (e), pOligomer IFNα S80A2).

④ The pOligomer IFNα S80A2 is digested with HindⅢ and S1 nuclease and then with Pst I to give the fragment whose one end is flush end beginning with TGT and another end is Pst I cohesive end that contains IFNα S80A2 structural gene.

⑤ The fragment of Trp-promoter (②) is linked to the fragment containing IFNα S80A2 structural gene (④) with T4 DNA ligase to provide pTrp-Sn-IFNα S80A2 (Fig. 7 (f)).

Ⅲ Transformation and Expression of IFNα

According to the expression vector employed, the IFNα expression plasmid provided in Ⅱ is introduced into an appropriate microorganism. In case using E. coli the transformation preferably achieved in accordance with the method of Hanahan et al, (J. Mol. Biol. 166, 557-580 (1983)). The resulting transformant is cultivated according to the usual method and the desired IFNα is separated from the culture and purified as occasion demands.

Example

The present invention is exemplified by the following example but is never restricted by the example.

Example 1.

Ⅰ. Preparation of cDNA of IFNα S80A2

(a) Induction of IFNmRNA in Ball-1 Cell

Into growth medium (RPMI 1640 medium containing 10 % fetal calf serum) human lymphoblast cells are inoculated by $1\text{-}2\times10^5$ cells/ml and then incubated in CO_2 incubator at 37°C for 3-4 days. After suspended to 8×10^5 cells/ml in growth medium containing 1 mM butyric acid, the cells are incubated at 37 °C for 48 hours and centrifuged by Sakuma 10B-2 rotor at 1200 rpm for 20 minutes. The resulting cells are suspended in growth medium (pH 7.2) containing 10 mM HEPES* to 5×10^6 cells/ml and treated with 100 IU/ml of IFNα with stirring at 100 rpm in a revolving incubating flask. After addition of 500-1000 hemagglutinin units/ml of Sendai virus (Cantell strain) the cells are incubated for 6-10 hours and collected by centrifugation by Sakuma 10B-2 rotor at 1200 rpm for 20 minutes.

* N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

(b) Preparation and Measurement of IFNmRNA

In order to collect cells when the amount of IFNmRNA therein reaches to maximum, a change of the production of IFN with the passage of time after the induction with Sendai virus is investigated (Table 8). After the induction with the virus, the cells incubated for 7, 8 or 9 hours are collected from each fraction to prepare mRNA. In order to estimate the rough amount of IFNmRNA in the obtained mRNAs, the mRNAs are injected into Xenopus oocyte according to the method of Cavalieri et al (Proc. Natl. Acad. Sci. 74, 3287 (1977)). After the oocytes are incubated at 20 °C for 48 hours, IFN activity in the medium is measured.

The IFN in the oocyte incubation medium is measured through Cell Pathologically Effect (CPE) inhibition activity in MDBK cell challenged with vesicular stomatits virus. The result is shown in Table 1.

Table 1

mRNA	IFN titer (u/μg mRNA)
Lot 48 (7 hr.)	275
Lot 49 (8 hr.)	250
Lot 50 (9 hr.)	49

From the results of Table 1, it is recognized that the cells incubated for 7 hours after induced with the virus contain a lot of IFNmRNA. In order to condense the IFNmRNA, mRNA prepared from the cells (4×10^9cells) at 7 hours after the induction with the virus is fractionated by 5-20 % sucrose gradient centrifugation, a portion of each fraction is injected into Xenopus oocyte to investigate the IFN activity, and fractions around the 12S from which IFNmRNAs are always obtained is separated.

(c) Synthesis and Cloning of cDNA

The synthesis of the first strand cDNA by Okayama-Berg's method using 12S fraction mRNA is carried out in 50 μl of reaction mixture containing 1.5 μg of mRNA, 50 mM of Tris-HCl (pH 8 .1), 50 mM of NaCl, 10 mM of $MgCl_2$, 10 mM of DTT, 0.2 mg/ml of bovine serum albumin (BSA), 220 u/ml of RNase inhibitor derived from human placenta, 5 μCi of (α-^{32}P) dCTP and 2 mM of dATP, dCTP, dGTP and dTTP. As primer is used vector-primer provided by linking about 60 deoxythymidine residues to one end of DNA fragment constituting the vector, and 3.5 μg of vector primer (corresponding to 1.7 pmol) is used so that the number of mRNA molecule is in excess. After the addition of 500 u/ml of reverse transcriptase, the reaction mixture is incubated at 37 °C for 60 minutes. To the mixture 2 μl of 0.5 M EDTA and 5 μg of poly (A)

are added to stop the reaction, then the mixture is extracted with phenol-chloroform. To the extract the same part of 4 M ammonium acetate (pH 5.0) and four parts of ethanol are added, and the mixture is cooled at -70 °C for 15 minutes and centrifuged for 10 minutes to give precipitate. The precipitate is dissolved in water again, and the procedure of the ethanol precipitation is carried out again. After washed with ethanol and lightly dried under reduced pressure, the precipitate is dissolved in water to advance to next step.

To the above precipitate (corresponding to 1.4 μg of vector-primer) is linked about 20 deoxycytidine residues at 3'-terminal using terminal deoxynucleotidyl transferase in 35 μl of reaction mixture containing 140 mM of sodium cacodylate, 30 mM of Tris-HCl (pH 6.8), 1 mM of $CoCl_2$, 0.1 mM of DTT, 0.1 mg/ml of BSA, 50 μCi of (α-^{32}P) dCTP and 50 μM of dCTP. To the reaction mixture is added 16 u of terminal deoxynucleotidyl transferase, incubated at 37 °C for 15 minutes and cooled rapidly to 0 °C to interrupt the reaction, while, to measure the uptake of [^{32}P] into TCA precipitate, 1 μl of the mixture is sampled for estimating the length of deoxycytidine residues. If the length is about 20 bases, the reaction is stopped then, but if the linked chain is too short, the reaction mixture is warmed to 37 °C again to be allowed to react for appropriate time after the addition of the enzyme. The reaction is stopped by adding 2 μl of 0.5 M EDTA, and the resultant is extracted with phenol-chloroform. To the extract are added one tenth parts of 3 M sodium acetate (pH 5.3) and 2.5 parts of ethanol, and then ethanol precipitation and ethanol washing is carried out in the same manner as noted above. The precipitate is lightly dried under reduced pressure and recovered by dissolved in water.

The recovered precipitate is digested with HindIII in 20 μl of reaction mixture containing 10 mM of Tris-HCl (pH 7.5), 60 mM of NaCl, 7 mM of $MgCl_2$ and 0.1 mg/ml of BSA. To the reaction mixture 12 u of HindIII is added, and the mixture is incubated at 37 °C for 60 minutes and extracted with phenol-chloroform. The extract are precipitated by adding one tenth parts of 3 M sodium acetate (pH 5.3) and 2.5 parts of ethanol thereto. The precipitate is lightly dried under reduced pressure and dissolved in water to obtain cDNA corresponding to about 0.8 μg of vector-primer.

The sample corresponding to 0.07 μg (0.035 pmol) of vector-primer is incubated with 13 ng (0.07 pmol) of linker DNA prepared by linking about 20 deoxyguanosine residues to one end of DNA fragment in 5 μl of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.1 M NaCl at 65 °C for 2 minutes, then at 42 °C for 30 minutes, and the mixture is cooled to 0 °C. The following ingredients are added thereto to adjust the volume to 50 μl: 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1 mM β-NAD, 50 μg/ml BSA and 15 u of E. coli DNA ligase. Then the mixture is incubated overnight at 12 °C

The following ingredients are added to the reaction mixture so as to bring the specified concentration.: 40 mM of dATP, dCTP, dGTP and dTTP, 0.15 mM β-NAD, 10 u of E. coli DNA ligase (as additional part), 2.8 u of DNA polymelase I (Klenow Fragment) and 0.9 u of E. coli RNase H. The mixture is incubated at 12 °C for 1 hour and then at 25 °C for 1 hour.

By using this reaction mixture, E. coli K-12 (χ776 or HB101 strain) is transformed to form cDNA bank according to the method of Hanahan et al. (J. Mol. Biol. 166, 557-580 (1983)).

(d) Preparation of ^{32}P-Oligodeoxynucleotide Probe

As a probe for screening human IFNα cDNA clone, two kinds

of mixed probes consisting of 16 bases in chain length are prepared.

① (5')AGATCACAGCCCA(C/G)AG

② (5')AGATTACAGCCCA(C/G)AG

According to the Goeddel's report (Nature 290, 20-26, (1981)), these sequences are complementary to 62th to 77th sequence from ATG of each subtype cDNA of human IFNα. However, there is no base sequence whose length is more than 14 bases that is common in all subtypes. Therefore, we synthesize 2 types of 16-mer which are mixed type having 2 kinds of base at one site and are different at one site from one another in order to cover cDNAs of all IFNα subtypes.

The labeling of oligodeoxynucleotide with ^{32}P is achieved by phospholylating with (γ-^{32}P)ATP and T4 polynucleotide kinase according to the method of Wallace et al (Nucl. Acids Res. 6, 3543-3557 (1979)). Moreover, (γ-^{32}P)ATP and T4 polynucleotide kinase which have not reacted is removed by gel filtration with Sephadex G-25.

(e) Screening of cDNA Clone with ^{32}P-Synthesised Oligodeoxynucleotide

The clone containing human IFNα cDNA is detected from transformant resistant against ampicillin obtained by Okayama-Berg's method according to colony hybridization (M. Grunstein et al, Proc. Natl. Acad. Sci. USA 72, 3961-3965 (1975)).

On 125 sheets of nitrocellulose filter are formed 10,000 colonies, bacteriolysised with alkali and fixed after DNA denaturation. After pretreated with 4×SSC*, 10×Denhardt solution (Biochem. Biophys. Res. Comm. 23, 641-646 (1966)) and 100 μg/ml

of E. coli DNA at 60 °C for 4 hours, the filters are hybridized at 35 °C for 15 hours with ^{32}P-synthesized-oligodeoxynucleotide (chain length 16) probe (5×10^5 cpm/filter) which is added to the newly prepared above-mentioned solution. After washed twice with 4×SSC at 4 °C for 15 minutes and air-dried, the filters are exposed at -70 °C to Kodak XAR-5 X-ray film by using Dupont lightening plus intensifying screen.

(* 1×SSC contains 0.15 M NaCl and 0.015 M sodium citrate (pH 7.9))

(f) Analysis of cDNA of IFNα

Each plasmid DNA is prepared from 79 clones hybridized with synthetic oligonucleotide probe. Firstly, the plasmids are digested by Pst I which cuts two sites of the vector to provide linear DNA, then 60 clones having insertion cDNA whose length is more than about 800 base pairs enough to contain human IFNα cDNA are selected.

Based on restriction enzyme map for each subtype of already known human IFNα, the subtype which each clone belongs to is presumed by making restriction maps for 60 clones according to the modified Southern hybridization, and then unknown clones and an already known clone (IFNαS80A2) are picked up.

Base sequence of cDNA clone (IFNαS51B10 and αS17H9) quite different from known ones is determined. Base sequence encoding each mature interferon and amino acid sequence deduced from the base sequence are shown in Fig.1-Fig.3. The main restriction map of each cDNA are shown in Fig.4-Fig.6.

Ⅱ. Preparation of Expression Plasmid

(g) Preparation of Expression Plasmid for IFNαS80A2

(ⅰ) Example of Using Synthesized Oligonucleotide Adaptor

① Expression vector (pTrp-promoter vector)(Fig. 7(b)) that

Trp-promoter-operater as promoter and SD sequence of E. coli are inserted into plasmid pBR322 at Cla I cutting site is used. This expression vector is digested with both Cla I and Acc I and the fragment of Cla I -Acc I which contains Trp-promoter is separated by polyacrylamide gel electrophoresis and cut out from the gel. The gel piece is broken in 10mM Tris-HCl (pH8) and 1mM EDTA and the supernatant is collected and precipitated with ethanol to recover DNA fragments. On the other hand, plasmid pIFNα S80A2 (Fig. 7(a)) is digested with both Acc I and Sau96I and the fragment containing IFN structural gene is separated by the gel electrophoresis in the same manner. This fragment is linked to the above fragment carrying Trp-promoter with T4 ligase and the resulting fragment linked at Acc I end is separated again by the gel electrophoresis.

② Plasmid pIFNα S80A2 is digested by Sau3AI and 176 bp fragment having Sau96 I site is separated by the gel electrophoresis (Fig. 7(a)). This fragment is digested by Sau96I to provide a mixture of 34 bp fragment and 142 bp fragment having Sau3AI end and Sau96I end.

③ Sau3AI cuts pIFNα S80A2 between the codon encoding the first amino acid and the codon encoding the second amino acid of N-terminal of mature IFN. Therefore, such synthesized oligodeoxynucleotide adaptor (5')CGATACATGTGT and (5')GATCACACATGTAT are prepared as has the codon TGT which encodes cysteine the first N-terminal amino acid and which is lost by Sau3AI digestion and the initiation triplet ATG for initiation of translation necessary in expression by E. coli.

④ The Trp-promoter-linked IFN structural gene fragment (①), the mixture of 34 bp fragment and 142 bp fragment (②) and the synthesized deoxynucleotide adaptor provided by annealing the

two fragments (③) are linked with T4 ligase. With the resulting recombinant DNA is transformed E. coli K-12 according to the method of Hanahan et al.

The transformant is selected on a plate containing ampicillin. From the resulting colonies resistant against ampicillin are selected a few colonies, from which plasmid DNA is isolated. The presence of desired fragment is confirmed by the restriction enzyme analysis. The provided plasmid is named pIrp-IFNα S80A2 (Fig. 7(c)). Moreover, the extract of E. coli carrying this plasmid has antiviral activity as noted later.

(i) Example of Using ATG Vector

① The above pIrp-promoter vector (Fig. 7(b)) is digested by both Cla I and Pst I and the fragment carrying Trp-promoter is isolated by the gel electrophoresis. Plasmid pBR322 is digested with both Pst I and EcoRI and the longer fragment is isolated by the gel electrophoresis. These two fragments and annealed synthetic deoxynucleotide oligomer (Sn) consisting of (5')CGATACTATATG and (5')AATTCATATAGTAT (n=11) prescribing SD-ATG are linked with T4 ligase. E. coli K-12 is transformed with the resulting recombinant DNA according to the method of Hanahan et al (the same as noted above).

The transformant is selected on a plate containing ampicillin and a few colonies are selected therefrom. The completion of preparation of ATG vector is confirmed by the restriction enzyme analysis of plasmid DNA isolated from the selected colonies.

② The above ATG vector is digested with EcoRI and then the EcoRI cohesive end is digested with S1 nuclease. After phenol-chloroform extraction and ethanol precipitation, the resultant is digested by Pst I and a fragment carrying Trp-promoter is separated by the gel electrophoresis. The fragment has Pst I

cohesive end, SD-ATG prescribed by Sn and flush end as a coding chain ends in ATG.

③ In the same manner as in (i), plasmid pIFNα S80A2 is digested with both Acc I and Sau96I to give a fragment having IFNα structural gene. Plasmid pBR322 is digested with both Cla I and Acc I and the longest fragment isolated by the gel electrophoresis is linked to the above DNA fragment at Acc I cutting site with T4 ligase. In the same manner as in (i), Sau3AI fragment (176 bp) of plasmid pIFNα S80A2 is digested with Sau96 I to give a mixture of 34 bp and 142 bp.

On the other side, such synthetic deoxynucleotide oligomers, (5')CGAAGCTTGT and (5')GATCACAAGCTT, are prepared as having the codon TGT at end which encodes the first amino acid (cysteine) of N-terminal of mature IFNα and which is lost by Sau3AI digestion and introducing HindⅢ cutting site just before TGT.

The annealed above oligomers, the above pBR322-IFNα structural gene-linked fragment and the mixture of 34 bp and 142 bp are linked with T4 ligase. E. coli K-12 is transformed with the resulting recombinant DNA in the same manner as in (i). In the same way as in (i), plasmid DNA is separated and the completion of preparing the desired plasmid is confirmed through the restriction enzyme analysis. The resulting plasmid is named pOligomer-IFNα S80A2 (Fig.7(e)).

④ The pOligomer-IFNα S80A2 prepared above is digested with Hind Ⅲ and then the Hind Ⅲ cohesive end is digested by S1 nuclease. After phenol-chloroform extraction and ethanol precipitation, the resultant is digested by Pst I and a fragment having IFNα structural gene is separated by the gel electrophoresis. The resulting fragment carrying IFNα structural gene has Pst I cohesive end and flush end as a coding chain begins

with IGT.

⑤ The fragment carrying Trp-promoter prepared in ② is linked to the fragment carrying IFNα structural gene prepared in ④ and E.coli K-12 is transformed with the resulting recombinant DNA in the same manner as noted above.

Transformant is selected on a plate containing ampicillin. A few colonies are selected from the resulting colonies resistant against ampicillin and each clone is multiplied. The antiviral activity in the extract of the E. coli is measured in a manner noted later and the transformants having the antiviral activity are recovered. The base sequence of the plasmid held by these transformants is analyzed and the each linkage of promoter, SD-ATG and IFN structural gene is confirmed to be a desired linkage. The plasmid recovered from these transformants is named pTrp-Sn-IFNα S80A2 (Fig. 7(f)) corresponding to the synthesized nucleotide oligomer Sn contained in ATG vector.

A standard strain of E. coli K-12 C600/pIFNα S80A2 prepared by transformation has been deposited as FERM P-7745 since July 25, 1984 in the Fermentation Research Institute Agency of the Industrial Science & Technology at Yatabe-machi, Tsukuba-gun, Ibaraki Pref. Japan.

Example 2

(a) Preparation of Expression Plasmid for IFNα S17H9 and IFNα S51B10

From two kinds of cDNA clone (IFNα S17H9 and IFNα S51B10) prepared in the above example 1-I-f is prepared each expression plasmid in the same manner as in example 1. Since both IFN structural genes have similar restriction enzyme cutting sites to one another as shown in Fig. 5 and 6, procedures for preparing the expression plasmids are almost the same as one another. Therefore,

a method for preparing both expression plasmids is shown below.

(i) Example of Using Synthesized Oligonucleotide Adaptor

By the method mentioned in example 1-(g)-(i) Trp-promoter vector (Fig. 7(b)) is digested with Cla I and Pst I and a Cla I - Pst I fragment having Trp-promoter is isolated.

On the other hand, plasmid pIFNα S17H9 or pIFNα S51B10 is digested with both Pst I and Xba I and about 2.3kbp fragment carrying the latter half of IFN structural gene is separated by the gel electrophoresis in the same way noted above. This fragment is linked to the previously separated fragment having Trp-promoter with T4 ligase and the fragment linked at Pst I site is isolated again by the gel electrophoresis.

Then, plasmid pIFNα S17H9 or pIFNα S51B10 is digested with Xba I and next partially with Sau3AI and a 245 bp fragment having the former half of IFNα structural gene is isolated by the gel electrophoresis (see Fig. 9 (a)).

This 245 bp fragment, the above Cla I -Xba I fragment having Trp-promoter and annealed synthetic oligodeoxynucleotide adaptor, (5')CGATACATGTGT and (5')GATCACACATGTAT, having initiation codon ATG and TGT encoding cysteine the amino acid of N-terminal of IFNα are mixed and linked with T4 ligase. With the resulting recombinant DNA is transformed E. coli K-12 according to the method of Hanahan et al.

The transformant is selected on a plate containing ampicillin. A few colonies are selected from the resulting colonies resistant against ampicillin, and plasmid DNA is isolated therefrom. The presence of the desired fragment is confirmed by the restriction enzyme analysis. The resulting plasmids are named pIrp-IFNα S17H9 and pIrp-IFNα S51B10, respectively (Fig. 9(c)). The extract of E. coli carrying this plasmid has antiviral

activity as mentioned later.

(i) Example of Using ATG vector

① ATG vector (Fig. 7(d)) is prepared in the same manner as in example 1-(g)-(i)-① and digested with EcoRI, S1 nuclease and Pst I in the same way as in the same-② to give DNA fragment having Pst I cohesive end, SD-ATG prescribed by Sn and flush end as coding chain ends in TAG.

② Pst I -Xba I fragment of plasmid pIFNα S17H9 or pIFNα S51B10 is prepared in the same manner as in (i). Plasmid pBR322 is digested with both Cla I and Pst I and the shorter Cla I -Pst I fragment is separated therefrom and linked to the above fragment at Pst I site. On the other hand, Sau3AI-XbaI fragment consisting of 245 bp is prepared from plasmid pIFNα S17H9 or pIFNα S51B10 in the same manner as in (i).

③ On the other side, synthetic deoxynucleotide oligomers, (5')CGAAGCTTGT and (5')GATCACAAGCTT, which have the codon TGT at the end which encodes the first amino acid (cysteine) of N-terminal of mature IFNα and which is lost by Sau3AI digestion and introduce HindⅢ cutting site just before TGT, are prepared.

④ The above oligomer annealed, the fragment of pBR322-IFNα structural gene prepared in above ② and 245 bp Sau3AI-Xba I fragment of IFNα S17H9 (or α S51B10) are linked with T4 ligase. With the resulting recombinant DNA is transformed E. coli K-12 in accordance with the method of Hanahan et al.

A plasmid is prepared from the transformant in the same way as in (i) and subjected to the restriction enzyme analysis to be confirmed that the desired plasmid is prepared.

The plasmids provided are named pOligomer-IFNα S17H9 and pOligomer-IFNα S51B10, respectively (Fig. 9(c)).

⑤ The plasmid prepared in above ④ is digested with HindⅢ

and the Hind Ⅲ cohesive end is digested by S1 nuclease. After the phenol-chloroform extraction and the ethanol precipitation, the resultant is digested with Pst I and the fragment carrying IFNα structural gene is separated by the gel electrophoresis. The fragment carrying IFNα structural gene has Pst I cohesive end and flush end as a coding chain begins with TGT.

⑥ The fragment carrying Trp-promoter provided in above ① is linked to the fragment carrying IFNα structural gene provided in above ⑤ with T4 ligase and with the resulting recombinant DNA is transformed E. coli K-12 (C600) in the same manner as noted above.

Transformant is selected on a plate containing ampicillin. A few colonies are selected from the resulting colonies resistant against ampicillin and each clone is amplified. The antiviral activity in the extract of the E. coli is measured in a manner noted later and the transformants having the antiviral activity are recovered. The base sequence of the plasmid held by transformants is analyzed and the each linkage of promoter, SD-ATG and IFN structural gene is confirmed to be a desired linkage. The plasmids recovered from these transformants are named pIrp-Sn-IFNα S17H9 and pIrp-Sn-IFNα S51B10, respectively (Fig.9 (d)) corresponding to the synthesized nucleotide oligomer Sn contained in ATG vector.

The transformants provided in the above procedure are named Escherichia coli K-12 C600/pIFNα S17H9 and Escherichia coli K-12 C600/pIFNα S51B10 , respectively, which have been deposited as FERM P-7766 and FERM P-7767, respectively, since August 8, 1984 in the Fermentation Research Institute Agency of the Industrial Science & Technology and have been transfered to the deposition under the Budapest Treaty with accession No.FERM BP-840 and FERM

BP-841, respectively, since July 11, 1985.

Effect of the Invention

I. Expression of IFNα S80A2

(i) Analysis of the Production from Plasmid Gene by Using In Vitro Transcription-Translation System.

Zubay et al reported that protein encoded by plasmid DNA can be produced in in vitro transcription-translation system by using E. coli extract (Methods in Enzymology 65, 856-877 (1980)).

The recombinant plasmid pTrp-IFNα S80A2 having Trp-promoter provided above is allowed to react in the presence of ^{35}S-methionine by using in vitro transcription-translation system kit (Amersham) according to the manual. The reaction production is analyzed by 16% SDS-polyacrylamide gel electrophoresis (Laemmli, Nature 227, 680-685 (1970)).

As a result, only polypeptide (MW c.a.20,000) presumed to be interferon and a small amount of the production of ampicillin-resistant gene are detected. Antiviral activity in the reaction mixture of this in vitro transcription-translation system is measured (according to the measurement method noted later) and about 100,000 u/ml of IFN is detected. The band of MW c.a. 20,000 is extracted from the gel and the IFN activity of the extract solution is measured to be recognized as positive.

(i) Expression of IFNα S80A2 in E. coli

E. coli K-12 C600 is transformed with plasmid pTrp-IFNα S80A2 (Fig. 7(c)) prepared above according to the method of Hanahan et al and the colonies growing on a plate containing 40μg/ml ampicillin are collected at random.

Next, each colony is cultured overnight in LB(Luria-Bertani) medium containing 40μg/ml ampicillin and 0.005 ml of this culture is inoculated into 5 ml of M9 medium supplemented 0.5% glucose,

0.5% casamino acid and 40μg/ml ampicillin and incubated at 37°C for 8 hours, and 5 ml of this culture is centrifuged. The resulting cell pellet, to which is added 2 ml of PBS (phosphate buffered saline) buffer solution supplemented 1% SDS, is destroyed by sonication to provide E. coli extract.

In determining a titer of IFN, a value determined by the dye uptake method employing Sindbis virus and FL cell (Protein, Nucleic acid and Enzyme (extra issue) 25, 355-363) is converted into international unit by standardizing the value with NIH human leukocyte IFN standard (G-023-901-527).

The titer of IFN in each E. coli extract prepared above is determined to be 10,000-50,000 u per 1 ml of the culture of E. coli. This IFN activity is neutralized only by the antibody against human IFNα but not at all by the antibody against human IFNβ or γ.

II Expression of IFNα S17H9 and α S51B10

The expression is carried out in E. coli with the recombinant plasmid having promoter prepared above.

For example, E. coli K-12 (C600) is transformed with the recombinant plasmid pTrp-IFNα S17H9 or pTrp-IFNα S51B10 (Fig.9 (b)) having Trp-promoter according to the method of Hanahan et al (noted above) and 10-20 strains are appropriately picked up from the colonies growing on a plate containing 40μg/ml ampicillin.

Next, each colony is cultured overnight in LB (Luria-Bertani) medium containing 40μg/ml ampicillin and 0.005 ml of this culture is transplanted into 5 ml of M9 medium supplemented 0.5% glucose, 0.5% casamino acid and 40μg/ml ampicillin and incubated at 37°C for 8 hours. The cell pellet provided by centrifuging 5 ml of this culture, to which is added 2 ml of PBS (phosphate buffered saline)buffer solution supplemented 1% SDS, is destroyed by

sonication to provide E. coli extract.

In determining a titer of IFN, a value determined by the dye uptake method employing Sindbis virus and FL cell (Protein, Nucleic acid and Enzyme (extra issue) 25, 355-363) is converted into international unit by standardizing the value with NIH human leukocyte IFN standard (G-023-901-527).

The titer of IFN in each E. coli extract prepared above is determined and the IFN activity per 1 ml of the culture of E. coli is shown in Table 2. The activity to mouse cell (LO) as well as human cell is determined and compared, and it is found that subtype S51B10 has activity to mouse cell but subtype S17H9 has not.

Table 2

subtype	IFN activity (IU/ml)	
	FL cell	LO cell (%)
S17H9	4,800 (100)	<2 (<0.004)
S51B10	38,000 (100)	5,000 (13)

IFNα S51B10 and IFNα S17H9 provided by this invention have antiviral and anti-tumor activity as other subtypes of IFNα and are useful compounds as a medicine for human and animal. The dose largely depends upon the subject and purpose of administration and the purity of administered IFN. However, these IFNαs may be administered to a normal adult man at a dose of about 10^6- 10^7 units (international unit) per day.

CLAIMS

1) Interferon α S51B10 or α S17H9.

2) A DNA encoding interferon α S51B10 or α S17H9.

3) The DNA of claim 2, which carries a sequence shown in Fig.2 or Fig.3.

4) A recombinant plasmid enabling an expression of interferon α S51B10 or α S17H9 in a host microorganism.

5) The recombinant plasmid of claim 4, which carries Trp-promoter.

6) The recombinant plasmid of claim 4, which is pIrp-IFNα S51B10, pIrp-Sn-IFNα S51B10, pIrp-IFNα S17H9 or pIrp-Sn-IFNα S17H9.

7) A microorganism transformed by a recombinant plasmid enabling an expression of interferon α S51B10 or α S17H9.

8) The microorganism of claim 7, which is Escherichia coli.

9) The microorganism of claim 7, which is E. coli K-12 C600/pIFNα S51B10.

10) The microorganism of claim 7, which is E. coli K-12 C600/pIFNα S17H9.

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Figure 1

```
        10         20         30         40         50         60
TGTGATCTGCCTCAGACTCACAGCCTGGGTAATAGGAGGGCCTTGATACTCCTGGCA...
C  D  L  P  Q  T  H  S  L  G  N  R  R  A  L  I  L  L  A  .

        70         80         90        100        110
ATGGGAAGAATCTCTCATTTCTCCTGCCTGAAGGACAGATATGATTTCGGATTCCCC...
M  G  R  I  S  H  F  S  C  L  K  D  R  Y  D  F  G  F  P  Q

       130        140        150        160        170        180
GAGGTGTTTGATGGCAACCAGTTCCAGAAGGCTCAAGCCATCTCTGCCTTCCATGAGATG
E  V  F  D  G  N  Q  F  G  K  A  Q  A  I  S  A  F  H  E  M

       190        200        210        220        230        240
ATCCAGCAGACCTTCAATCTCTTCAGCACAAAGGATTCATCTGCTGCTTGGGATGAGACC
I  Q  Q  T  F  N  L  F  S  T  K  D  S  S  A  A  W  D  E  T

       250        260        270        280        290        300
CTCCTAGACAAATTCTACATTGAACTTTTCCAGCAACTGAATGACCTAGAAGCCTGTGTG
L  L  D  K  F  Y  I  E  L  F  Q  Q  L  N  D  L  E  A  C  V

       310        320        330        340        350        360
ACACAGGAGGTTGGGGTGGAAGAGATTGCCCTGATGAATGAGGACTCCATCCTGGCTGTG
T  Q  E  V  G  V  E  E  I  A  L  M  N  E  D  S  I  L  A  V

       370        380        390        400        410        420
AGGAAATACTTTCAAAGAATCACTCTTTATCTGATGGGGAAGAAATACAGCCCTTGTGCC
R  K  Y  F  Q  R  I  T  L  Y  L  M  G  K  K  Y  S  P  C  A

       430        440        450        460        470        480
TGGGAGGTTGTCAGAGCAGAAATCATGAGATCCTTCTCTTTTTCAACAAACTTGCA...
W  E  V  V  R  A  E  I  M  R  S  F  S  F  S  T  N  L  Q  K

       490        500
GGATTAAGAAGGAAGGATTGA
G  L  R  R  K  D  *
```

Figure 2

```
        10         20         30         40         50         60
TGTGATCTGCCTCAGACCCACAGCCTGAGTAACAGGAGGACTTTGATGATAATGGCACAA
C  D  L  P  Q  T  H  S  L  S  N  R  R  T  L  M  I  M  A  Q

        70         80         90        100        110        120
ATGGGAAGAATCTCTCCTTTCTCCTGCCTGAAGGACAGACATGACTTTGGATTTCCTCAG
M  G  R  I  S  P  F  S  C  L  K  D  R  H  D  F  G  F  P  Q

       130        140        150        160        170        180
GAGGAGTTTGATGGCAACCAGTTCCAGAAGGCTCAAGCCATCTCTGTCCTCCATGAGATG
E  E  F  D  G  N  Q  F  Q  K  A  Q  A  I  S  V  L  H  E  M

       190        200        210        220        230        240
ATCCAGCAGACCTTCAATCTCTTCAGCACAAAGGACTCATCTGCTACTTGGGATGAGACA
I  Q  Q  T  F  N  L  F  S  T  K  D  S  S  A  T  W  D  E  T

       250        260        270        280        290        300
CTTCTAGACAAATTCTACACTGAACTTTACCAGCAGCTGAATGACCTGGAAGCCTGTATG
L  L  D  K  F  Y  T  E  L  Y  Q  Q  L  N  D  L  E  A  C  M

       310        320        330        340        350        360
ATGCAGGAGGTTGGAGTGGAAGACACTCCTCTGATGAATGTGGACTCTATCCTGACTGTG
M  Q  E  V  G  V  E  D  T  P  L  M  N  V  D  S  I  L  T  V

       370        380        390        400        410        420
AGAAAATACTTTCAAAGAATCACTCTCTATCTGACAGAGAAGAAATACAGCCCTTGTGCA
R  K  Y  F  Q  R  I  T  L  Y  L  T  E  K  K  Y  S  P  C  A

       430        440        450        460        470        480
TGGGAGGTTGTCAGAGCAGAAATCATGAGATCCTTCTCTTTATCAGCAAACTTGCAAGAA
W  E  V  V  R  A  E  I  M  R  S  F  S  L  S  A  N  L  Q  E

       490        500
AGATTAAGGAGGAAGGAATGA
R  L  R  R  K  E  *
```

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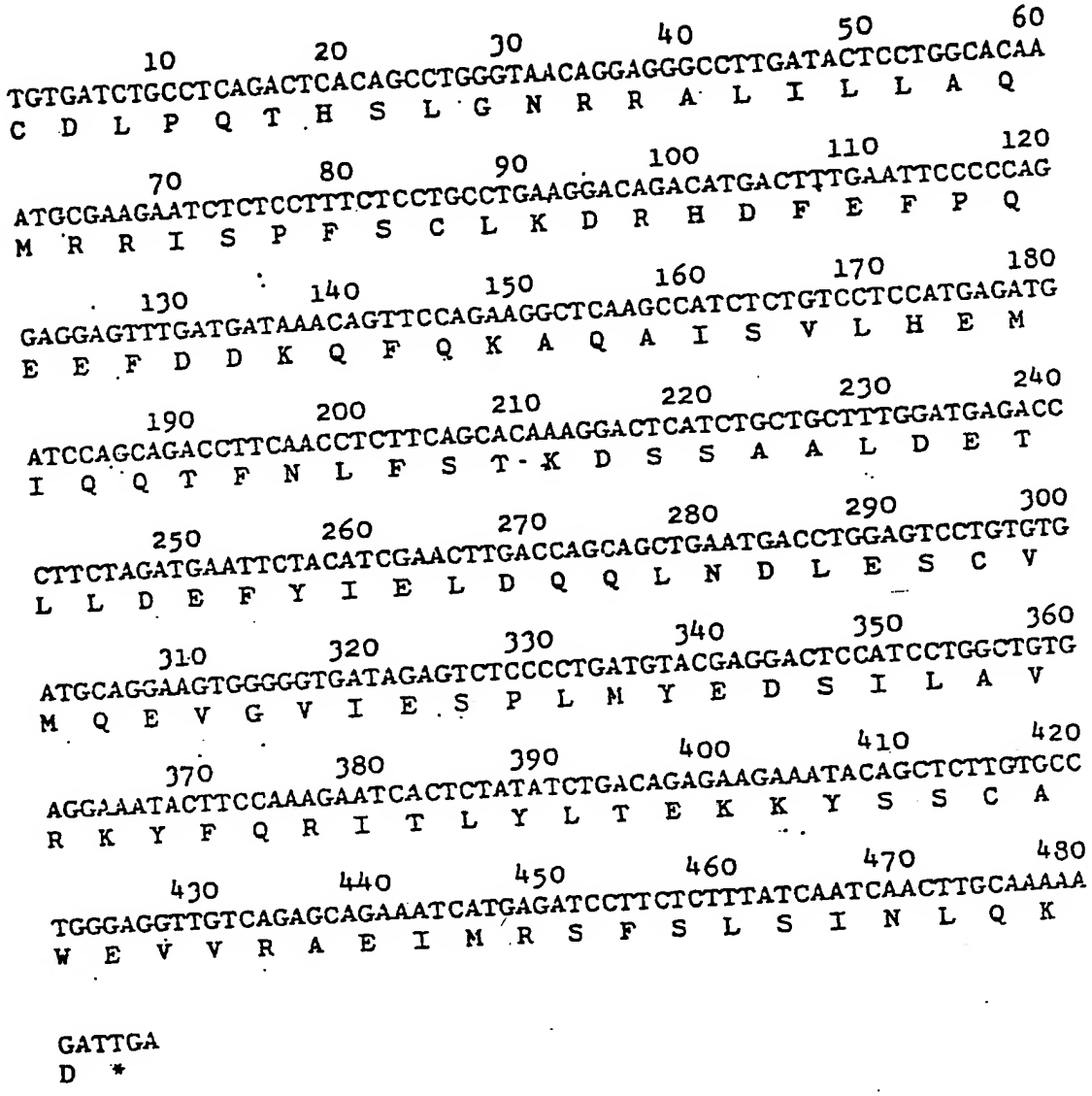
Figure 3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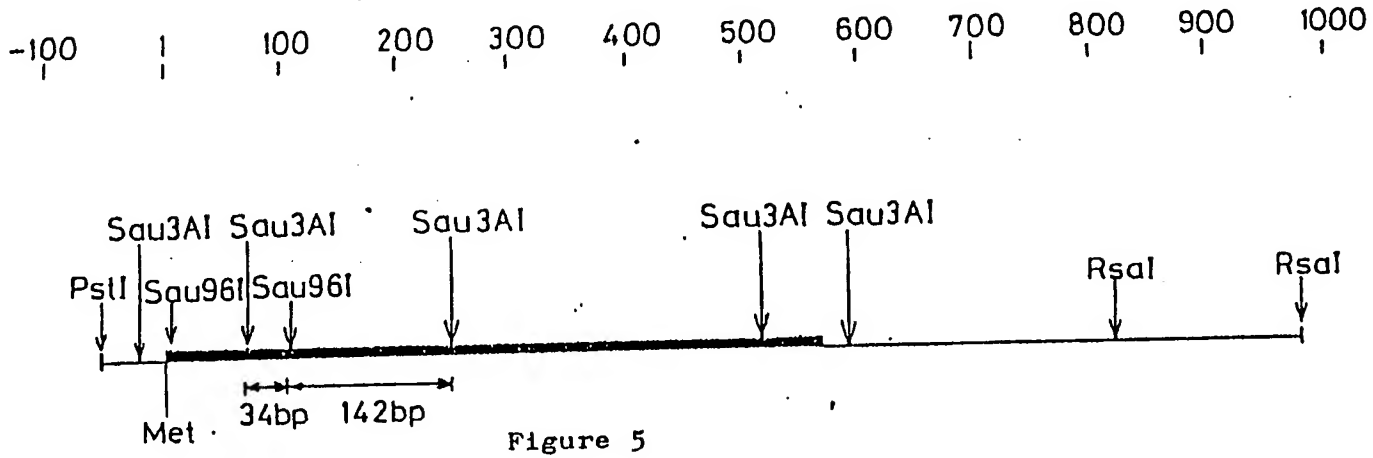
C D L P Q T H S L G N R R A L I L L A Q
70 80 90 100 110 120
ATGCGAAGAATCTCTCCTTTCTCCTGCCTGAAGGACAGACATGACTTTGAATTCCCCCAG
M R R I S P F S C L K D R H D F E F P Q
130 140 150 160 170 180
GAGGAGTTTGATGATAAACAGTTCCAGAAGGCTCAAGCCATCTCTGTCCTCCATGAGATG
E E F D D K Q F Q K A Q A I S V L H E M
190 200 210 220 230 240
ATCCAGCAGACCTTCAACCTCTTCAGCACAAAGGACTCATCTGCTGCTTTGGATGAGACC
I Q Q T F N L F S T-K D S S A A L D E T
250 260 270 280 290 300
CTTCTAGATGAATTCTACATCGAACTTGACCAGCAGCTGAATGACCTGGAGTCCTGTGTG
L L D E F Y I E L D Q Q L N D L E S C V
310 320 330 340 350 360
ATGCAGGAAGTGGGGGTGATAGAGTCTCCCCTGATGTACGAGGACTCCATCCTGGCTGTG
M Q E V G V I E S P L M Y E D S I L A V
370 380 390 400 410 420
AGGAAATACTTCCAAAGAATCACTCTATATCTGACAGAGAAGAAATACAGCTCTTGTGCC
R K Y F Q R I T L Y L T E K K Y S S C A
430 440 450 460 470 480
TGGGAGGTTGTCAGAGCAGAAATCATGAGATCCTTCTCTTTATCAATCAACTTGCAAAAA
W E V V R A E I M R S F S L S I N L Q K
GATTGA
D *

Figure 4



8/4

Figure 5

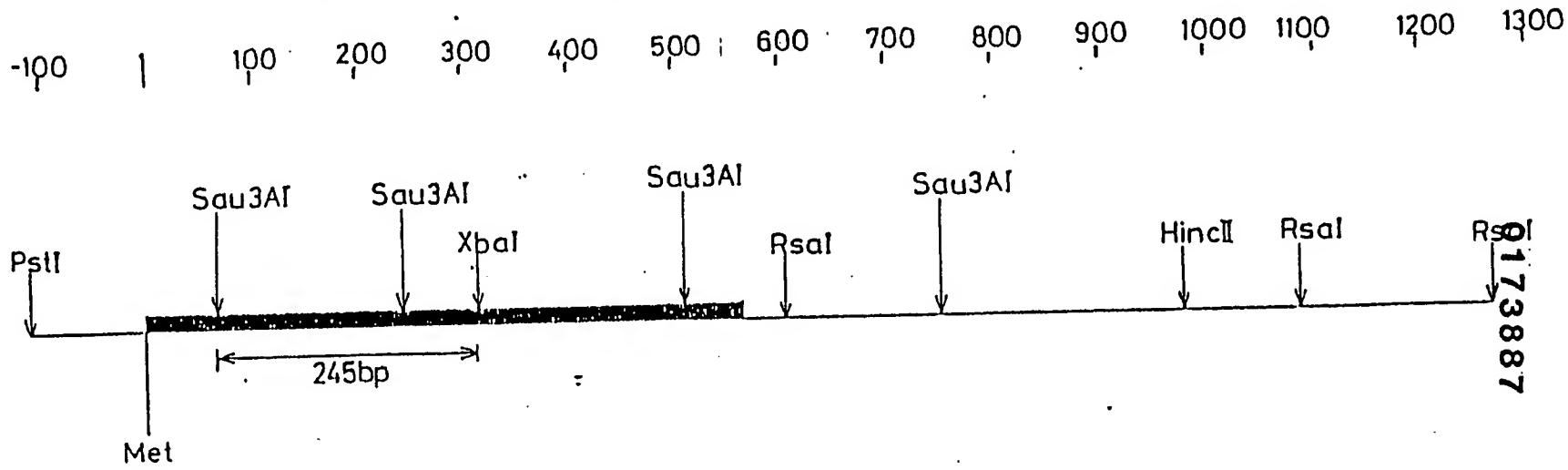


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Figure 6

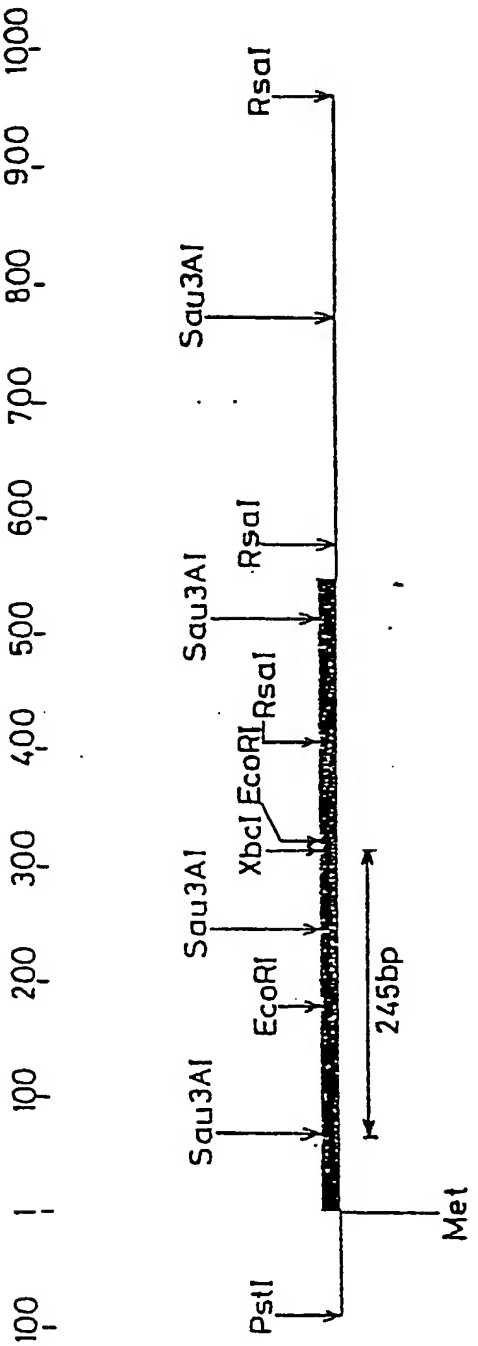

1000
900
800
700
600
500
400
300
200
100
1
100
RsaI
Sau3AI
Sau3AI
RsaI
Sau3AI
RsaI
EcoRI
XbcI
Sau3AI
EcoRI
Sau3AI
245bp
Met
PstI

Figure 7

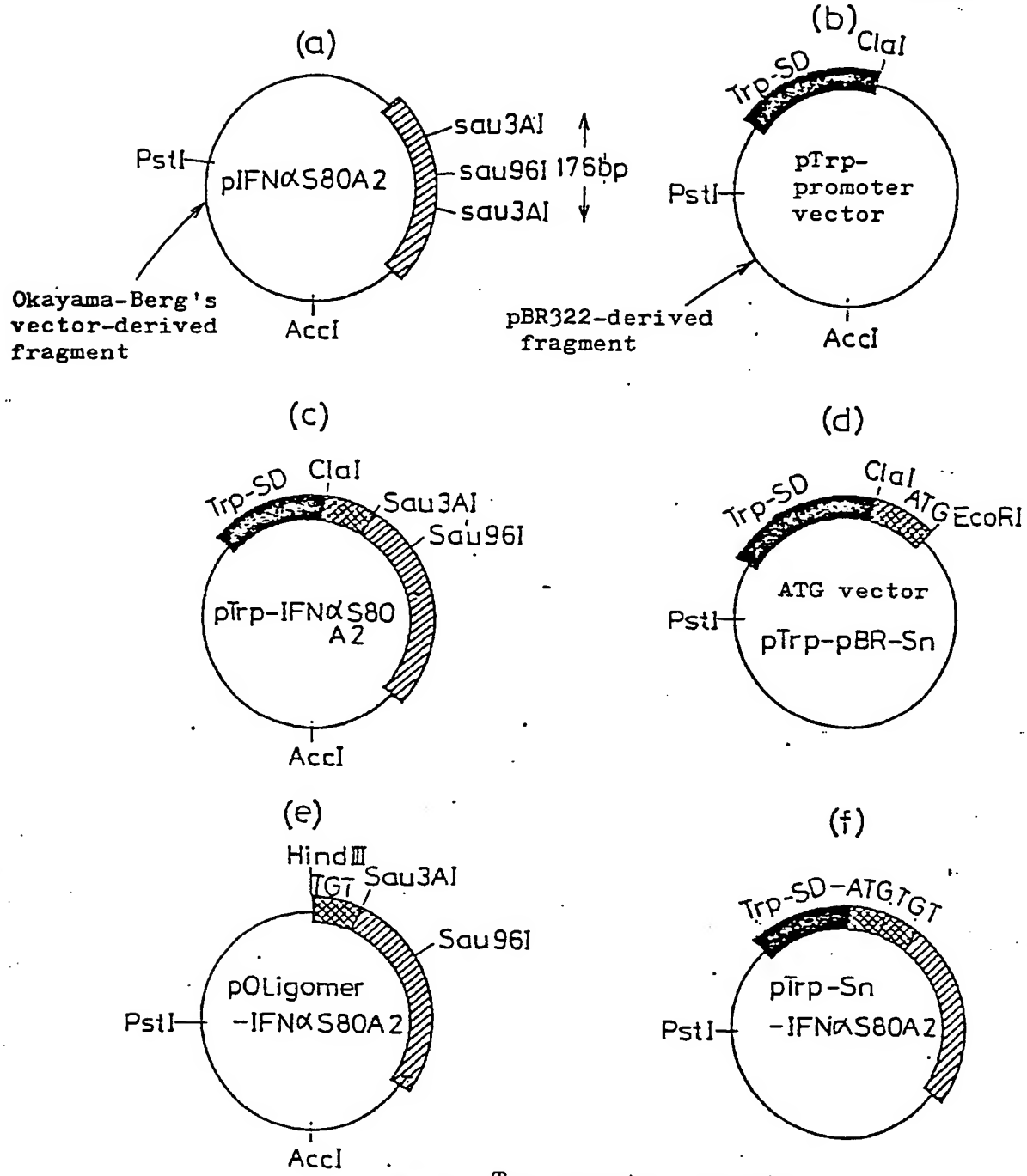

(a)
PstI
pIFNαS80A2
Sau3AI
Sau96I
176bp
Sau3AI
AccI
Okayama-Berg's vector-derived fragment
(b)
ClaI
Trp-SD
pTrp-promoter vector
PstI
AccI
pBR322-derived fragment
(c)
Trp-SD
ClaI
Sau3AI
Sau96I
pTrp-IFNαS80 A2
AccI
(d)
Trp-SD
ClaI
ATG
EcoRI
ATG vector
PstI
pTrp-pBR-Sn
(e)
HindIII
TGT
Sau3AI
Sau96I
pOLigomer -IFNαS80A2
PstI
AccI
(f)
Trp-SD-ATG TGT
pTrp-Sn -IFNαS80A2
PstI
Trp-promoter operator and Shine-Dalgarno sequence
Synthetic oligonucleotide adaptor or oligomer
IFNα cDNA-derived DNA

Figure 8

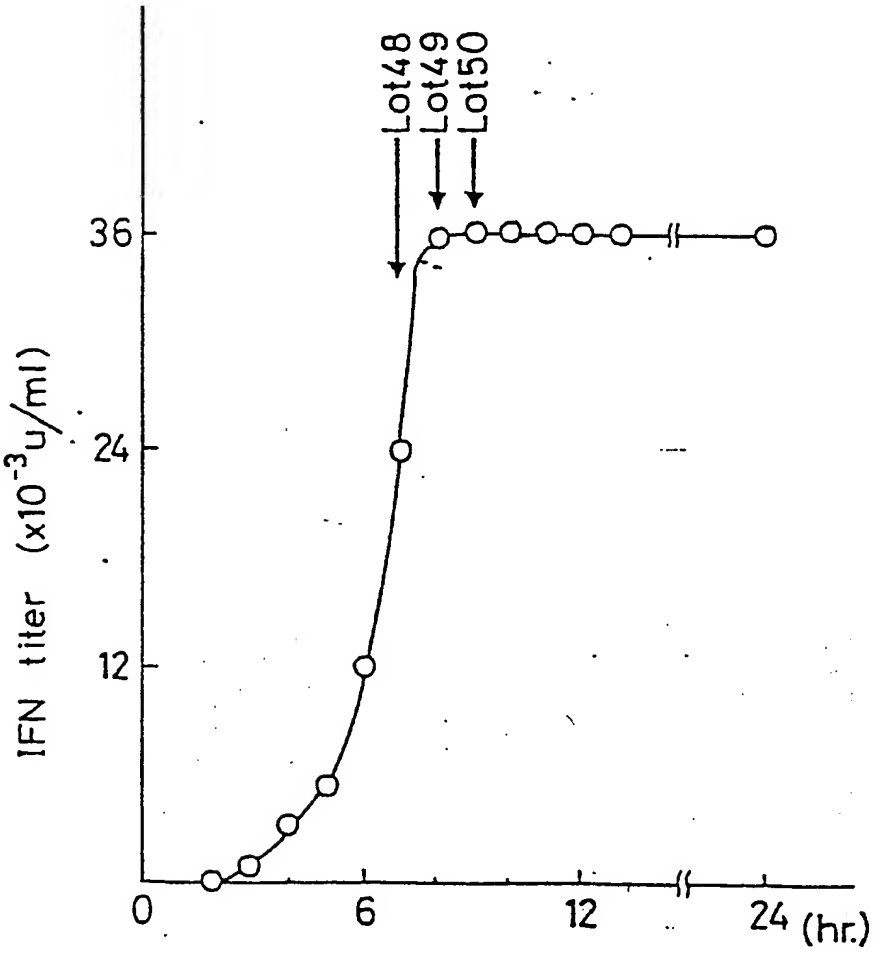
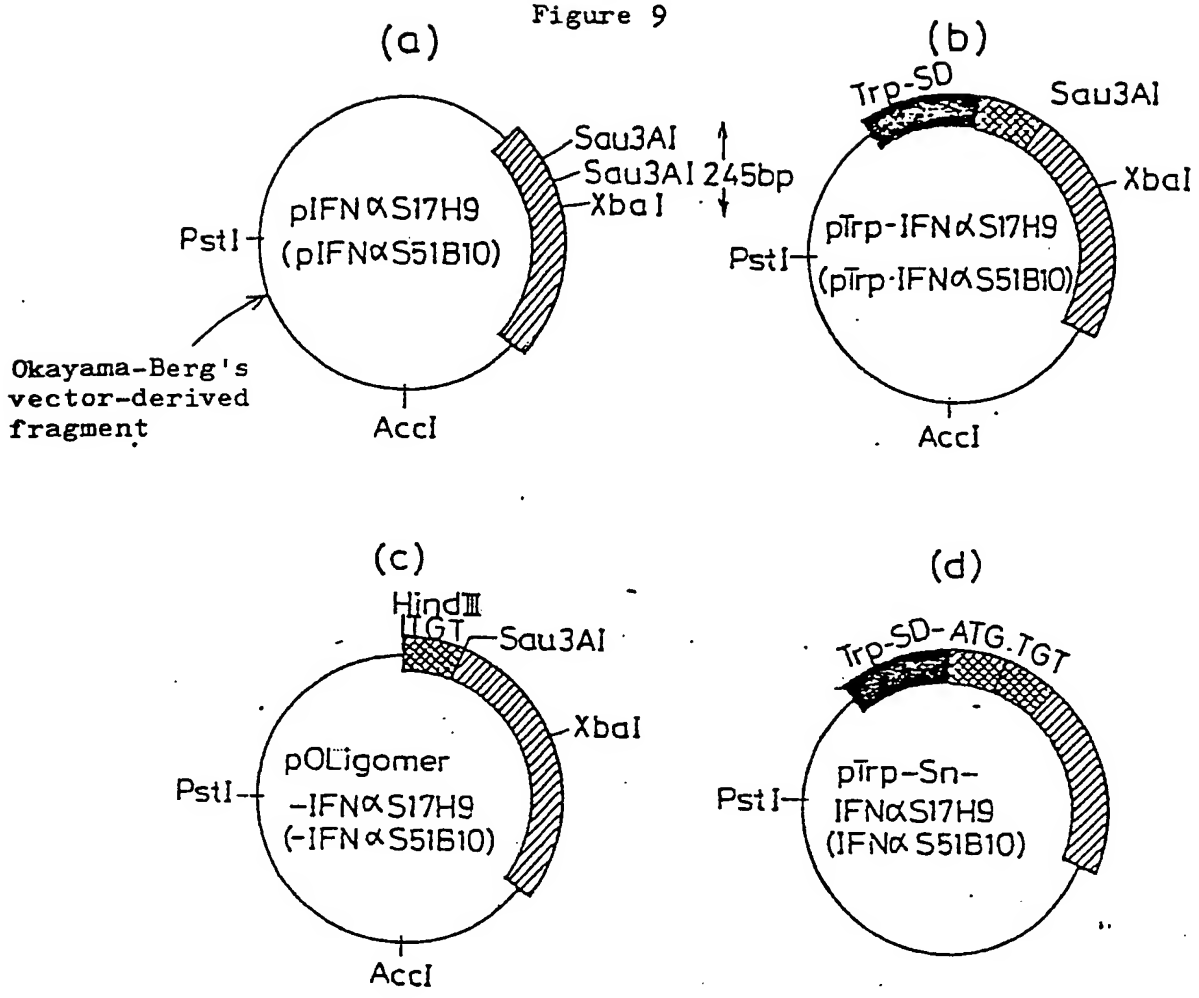


Figure 9



Trp-promoter operator and Shine-Dalgarno sequence

Synthetic oligonucleotide adaptor or oligomer

IFNα cDNA-derived DNA

European Patent Office

EUROPEAN SEARCH REPORT

0173887 Application number

EP 85110061.0

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl 4)
A	EP - A3 - 0 076 489 (CIBA-GEIGY) * Claims 1-45 * --	1-4,7, 8	C 07 K 15/26 C 12 N 15/00 C 12 N 1/20 C 12 P 19/34 //A 61 K 45/02 C 12 R 1:19
A	EP - A2 - 0 051 873 (GENENTECH) * Claims 1-15; page 6, lines 6-15 * --	1-8	
A	WO - A1 - 84/00 776 (CETUS) * Claims; page 9, lines 3-17 * --	1-8	
A	WO - A1 - 83/02 459 (CETUS) * Claims 1-17 * ----	1-8	

TECHNICAL FIELDS SEARCHED (Int. Cl 4)

C 07 K
C 12 N
C 12 P
A 61 K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	02-12-1985	BECKER

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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